4-2015

Cyclin-Dependent Kinase 5 Mediates Metastatic Melanoma Cell Invasion into a Brain Micro-Environment

Nicholas C. Borkey
University of Dayton, ncb32@case.edu

Cyclin-Dependent Kinase 5 Mediates Metastatic Melanoma Cell Invasion into a Brain Micro-Environment

Honors Thesis

Nicholas C. Borkey

Department: Biology

Advisor: John Letterio, M.D.

April 2015

Cyclin-Dependent Kinase 5 Mediates Metastatic Melanoma Cell Invasion into a Brain Micro-Environment

Honors Thesis

Nicholas C. Borkey

Department: Biology

Advisor: John Letterio, M.D.

April 2015

Abstract

While survival rates for localized melanoma are above 98%, metastatic melanoma proves to be an extraordinarily dreadful disease because of its notoriously poor prognosis resulting in less than 15% survival within 5 years. This is due to the fact that the disease is highly resistant to standard cancer therapies. With approximately 40% of melanoma patients progressing to brain metastasis, a greater understanding of the pathways mediating melanoma cell invasion into the brain must be achieved. In this study, a potentially key target often deregulated in melanoma and other cancers, Cyclin-Dependent Kinase 5 (CDK5), will be interrogated for its implications in metastatic melanoma cell invasion into the brain. Despite the observed deregulation of CDK5 in various cancers as well as human metastatic melanoma cell lines, there is currently minimal effort in defining its role in brain metastatic melanoma. This study approaches this problem in an innovative way by exploring Neurotrophin Receptors (NTR) signaling pathways involving CDK5. Specifically, activated Neurotrophin Receptors induce EGR-1, thus transcribing p35 and activating its obligate partner CDK5. Considering CDK5 is implicated in a variety of cellular processes such as survival and invasion, this pathway supports our central hypotheses that activation of CDK5 through Neurotrophin (NT) signaling is vital in melanoma cell invasion into the brain. In order to test this hypotheses, a transwell invasion assay utilizing human melanoma cells was used in order to mimic melanoma metastasis into a brain micro-environment in vitro. In order to implicate CDK5 in this study, neurotrophin induced invasion of human metastatic melanoma cells with wild-type expression of CDK5 were compared to cells with drug inhibited CDK5 as well as lentivirally transduced CDK5 knockout cells. Upon NT stimulation, metastatic melanoma cells containing active CDK5/p35 demonstrated a higher propensity to invade into the brain micro-environment. Moreover, simultaneous stimulation of metastatic

melanoma cells by multiple members of the neurotrophin family were shown to synergistically enhance invasion ability into a brain micro-environment when compared to stimulation by a single type of neurotrophin.

Acknowledgements

A major thank you goes out to all members of the Letterio Lab at the Case Comprehensive Cancer Center who helped me pursue this thesis. In particular, I would like to acknowledge Dr. Seunghwan Lim, Ph.D., and Dr. Tej Pareek, Ph.D.

Table of Contents

Introduction

In order to understand the purpose and data generated from this study, various pieces of background information must be clearly laid out. In general, this introductory section briefly explains the current treatment options for patients with brain metastatic melanoma (BMM). From here, the potentially therapeutic target named Cyclin-Dependent Kinase 5 (CDK5) will be described with regards to its role in metastatic melanoma that is supported by existing research. The next part of this introductory section details a family of proteins called neurotrophins that existing research shows may have a role in melanoma brain metastases. Finally, this introductory section will conclude by tying CDK5 to neurotrophins in the context of metastatic melanoma in order to set the stage for the experiments that compose this study.

Current Treatments for Patients with Brain Metastatic Melanoma

Over the past 50 years, the annual mortality due to melanoma in humans has increased more than any other form of cancer[1]. While survival rates for localized melanoma are above 98%, metastatic melanoma proves to be an extraordinarily dreadful disease because of its notoriously poor prognosis at less than 15% survival over 5 years in addition to the tendency of this disease to resist standard cancer therapy regiments[2]. One common site of metastasis observed in human melanoma is the brain, with approximately 40% melanoma patients progressing to brain metastatic melanoma (BMM)[3]. In order for brain metastasis to occur, melanoma cells must travel from the primary tumor site, migrate to the brain, invade across the blood brain barrier, and survive long enough to induce angiogenesis that will allow tumor cell proliferation to

occur in the brain tissue[4]. Only 0.01%-0.03% of melanoma cells will metastasize to any target organ, thus illustrating that a small proportion of cells can lead to immense problems within the body[5-9]. Currently, the primary treatment option for BMM patients is surgical resection, which carries the risk of damaging healthy brain tissue and oftentimes does not improve patient prognosis to a significant degree[4]. Furthermore, standard cancer treatments such as chemotherapy typically yield low response[10]. Recently, targeted therapies involving inhibition of key signaling intermediates in melanoma metastasis have shown promising results, but are often hindered due to toxicity[4]. One example of targeted therapies for BMM can be seen through BRAF inhibitors. The BRAF oncogene harbors a mutation in roughly 65% of melanoma patients, leading to constitutive activation of the MEK→ERK→EGF-1 pathway, and thus upregulation of p35, ultimately leading to constitutive activation of CDK5. BRAF pathway inhibitors such as dabrafenib and vemurafenib have been tested in a clinical setting with some success. Specifically, the BRAF inhibitor dabrafenib showed similar response in patients with and without prior treatment, indicating BRAF inhibition as a viable option for coupled treatments. In addition, the side effects of dabrafenib were relatively limited and included primarily fatigue and nausea. In this regard, certain BMM cases such as those with extensive extra-cranial metastasis may qualify for initial BRAF inhibition followed by surgical resection. However, an additional limitation for surgical resection is the necessary use of corticosteroids following the procedure, possibly leading to the impairment of coupled immunotherapeutic treatments. Even following surgical resection, patient prognosis is still poor and the procedure carries the potential to damage the brain. However,

treatments such as BRAF inhibition that can suppress tumor growth and allow for a more successful surgical resection demonstrate a promising avenue to pursue.

Whole brain radiotherapy is used to supplement or even replace the surgical procedure, although relying on whole brain radiotherapy as the primary treatment option is often reserved for cases involving larger tumors (>3cm) or an abundance of metastatic sites in the brain (>3)[4]. If possible, targeted radiotherapy is used, limiting the damaging effects to the brain incurred in whole brain radiotherapy.

Patients with BMM show poor response to standard chemotherapy due to a variety of factors that are not well understood. This may be due to the inability of the treatment to penetrate the blood-brain-barrier as well as tumor cell resistance to apoptosis via paracrine signaling from astrocytes near the tumor site[11-12]. Even those chemotherapies that are proven to penetrate the blood-brain-barrier are hampered by low response rates and short duration of response. However, chemotherapy coupled with whole brain radiotherapy has shown indications of improved patient prognosis.

Recently, immunotherapies have been shown to be relatively effective and safe in treating human metastatic melanoma. One such example, ipilimumab, functions to potentiate anti-tumor response and has been shown to be relatively effective in treating asymptomatic cases of BMM. In relation to this, patients with BMM who display higher levels of vascular endothelial growth factor (VEGF) in the blood have a poor response to the immunotherapy ipilimumab[4]. This is due to the fact that high levels of VEGF promotes angiogenesis and suppresses the anti-tumor response of immune cells, a process that is needed in order for tumors in the brain to grow. As a result of this, antiangiogenics

have been considered as a potential treatment option for patients with BMM. However, up to one fifth of patients treated with antiangiogenics experience hemorrhaging in clinical trials.

Overall, it is clear that current treatment options for patients with BMM leave a lot to be desired. However, the promise that novel treatments such as those seen with BRAF inhibitors demonstrate that downstream effectors in the BRAF pathway may prove to be worthwhile therapeutic targets. One such target is CDK5. This kinase has been implicated as a relevant target in a variety of cancers, yet has not been established as a therapeutic target for patients with BMM.

Cyclin-Dependent Kinase 5 in the Context of Metastatic Melanoma

When CDK5 was first studied, it was believed to be implicated mostly in neuronal function and development. However, numerous studies have since shown that this kinase is expressed in all tissues and plays a role in an extraordinary amount of disease pathologies and cell processes including but not limited to cell cycle, growth, migration and invasion. In fact, evidence supports that CDK5 is involved in both tumor development as well as metastatic processes. Not surprisingly then, CDK5 has been shown to be upregulated in numerous cancers. Specifically to melanoma, one study of 45 patients revealed an average of over a 21-fold increase in CDK5 expression in the tumor tissue [13]. To compound this, normal skin and nevus cells show no expression of CDK5, showing that melanoma tumor formation accompanies a rise in expression of CDK5 [14]. Melanoma cells have been shown to be responsive to CDK5 inhibition. Specifically, mouse models of melanoma have demonstrated reduced tumor growth in cohorts treated

with dinaciclib, a small molecule CDK inhibitor, when compared to cohorts not treated with a CDK inhibitor [15]. However, the utility of CDK5 inhibitors in treating BMM is not yet clear.

Neurotrophins in the Context of Metastatic Melanoma

Just over 25 years ago, nerve growth factor (NGF) was identified as a factor for neuronal survival[16]. This was the first in a family of survival factors that would go on to be classified as neurotrophins. However, not all neuronal populations were responsive to NGF, and thus the search for other factors led to the isolation additional members of this family of proteins; NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). These four neurotrophins are the only four found in mammals. Although neurotrophins are implicate in a variety of processes, this study is concerned primarily with the established role of neurotrophins in cell survival and differentiation[17]. These effects are due to pathways activated by the interaction between neurotrophins and several different cell-surface receptors that specifically bind one or more member in the neurotrophin family. There are two separate classes of these cell-surface receptors; the Trk family of receptors, which bind with higher affinity and specificity, and the p75 neurotrophin family receptor (p75NTR), which binds with low affinity and specificity[18]. While the p75NTR binds all mammalian neurotrophins, the Trk family of receptors, which includes TrkA, TrkB, and TrkC) bind only specific neurotrophins. Particularly, TrkA binds Nt-3 and NGF, TrkB binds BDNF, NT-3, and NT-4, and TrkC binds NT-3.

Although the role of neurotrophins was originally studied in the context of neuronal populations, neurotrophins also function in a variety of other tissues, namely in the skin. Evidence shows that these proteins function both in both an autocrine and paracrine manner in the skin, suggesting a probable role in the invasive capacity of metastatic melanoma. Not only have studies shown that neurotrophins promote melanoma cell survival, but also brain tissue adjacent to brain tumor cells secrete neurotrophins. Moreover, neurotrophins also induce cellular production of heperanase in melanoma cells, lending to brain invasive capacity[19].

Neurotrophins and CDK5 in Metastatic Melanoma

The skin and nervous system share a common neuroectodermal origin, supporting the possibility that melanoma has a high propensity to invade the brain because it provides a favorable environment for metastatic melanoma cells to thrive. However, the role of neurotrophins in metastatic melanoma has been studied relatively little compared to other cancers derived from the neural crest, despite the fact that cancers of this origin share oncogenic pathways [20, 21]. One such pathway can be seen in Figure 1. This pathway demonstrates an interconnection between neurotrophin signaling and CDK5 expression mediated by a variety of intermediates. In this way, it can be seen that stimulation of metastatic melanoma cells by neurotrophins can lead to increased invasion capacity via up-regulation of the obligate activator of CDK5 named p35. The following experiments

seek to elucidate this pathway and establish CDK5 as a promising therapeutic target in brain metastatic melanoma.

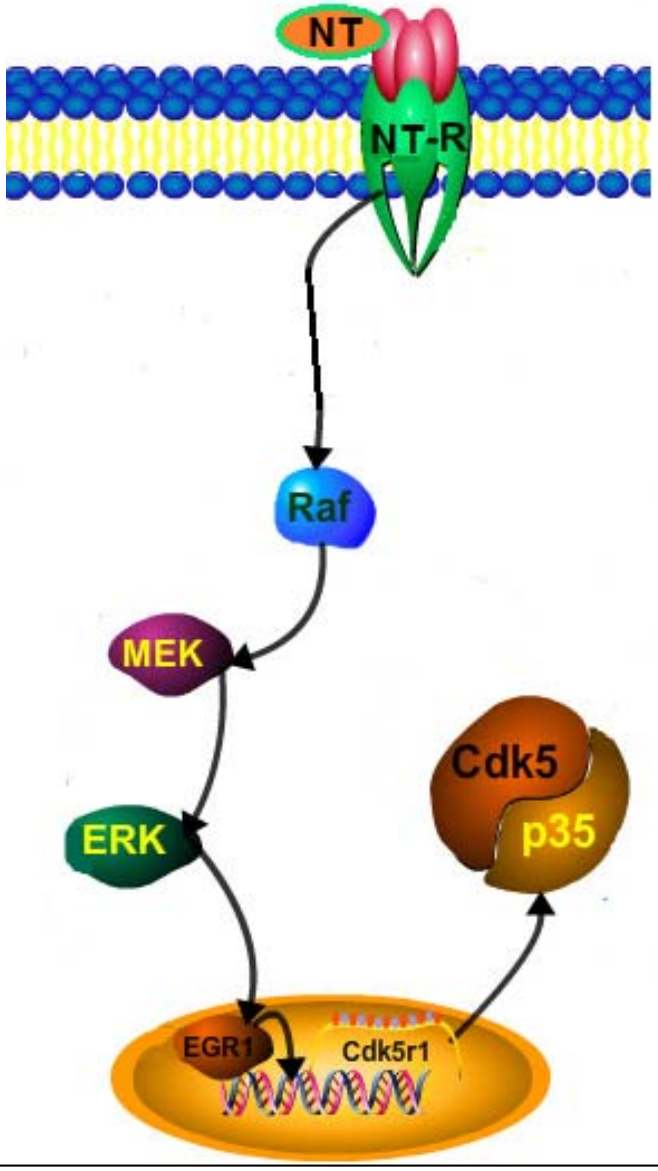

Figure 1 depicts a cartoon illustration of a NTR signaling pathway. Notably, NT binds NT-R, causing induction of EGR-1 and transcription of the CDK5 activator p35.

Methods

In order to implicate CDK5 in the melanoma brain metastasis, we planned two experiments involving reduction of CDK5 activity in metastatic melanoma cells for use in Matrigel transwell assays that mimic a brain micro-environment.

Experiment 1

The purpose of the first experiment was to demonstrate that the invasive capacity human metastatic melanoma cells into a brain micro-environment is proportional to CDK5 activity. In other words, higher CDK5 activity results in a higher propensity for invasion into a brain micro-environment. In order to accomplish this aim, a CDK5 inhibitor known as roscovitine was used in various concentrations to reduce CDK5 activity. The structure of roscovitine, which can be seen in figure 2, is a purine analog and thus competes for the ATP binding pocket on CDK5. By blocking CDK5 from binding ATP, the kinase loses its phosphorylating ability and is thus inactive. A cross-section of a single well in the 24 transwell plate that was used for this assay can be seen in figure 3. Each well in the transwell plate contains two halves which are separated from one another by a cell permeable Matrigel membrane. This Matrigel membrane contains structural proteins that function as an in

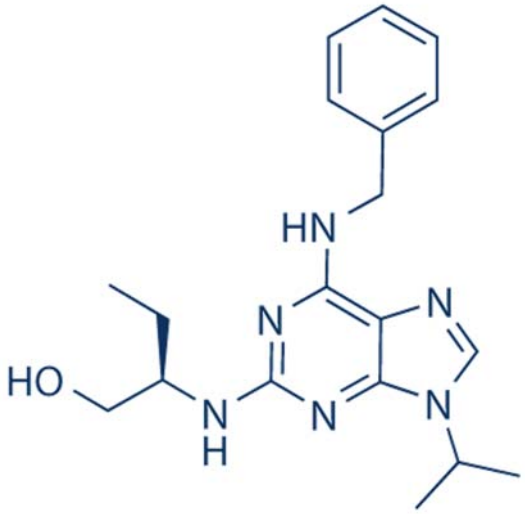

Figure 2 shows the chemical structure of the CDK5 inhibitor Roscovitine. This drug functions by occupying the ATP binding pocket

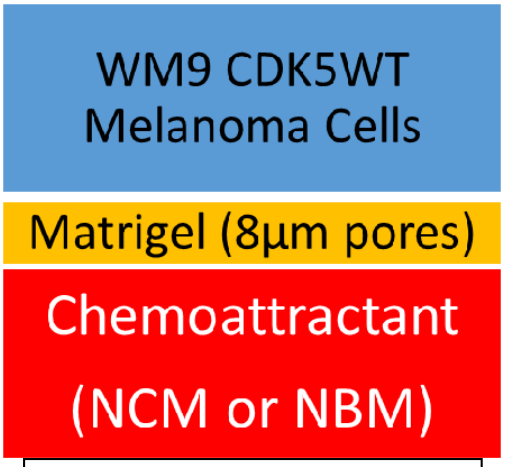

Figure 3 depicts a cross-section of a single well in the transwell plate used for experiment 1. Each well is separated by a Matrigel membrane. In the top half of each well are confluent WM9 CDK5WT cells +/- Roscovitine. In the bottom half of each well, either NCM or NBM functions as a

vitro model of a blood brain barrier. Cells are able to permeate this membrane by chemotactically migrating through 8 micron pores in the Matrigel in response to a chemoattractant contained in the bottom half of the transwell. In experiment 1, the top half of each well was seeded in serum-free RPMI media at a density of $2x10^5$ WM9 cells per well with wildtype CDK5 expression (CDK5WT). Then, Roscovitine was introduced into the top half of the well in various concentrations (0µM, 1µM, 5µM, and 10µM). At the same time, either neural conditioned media (NCM) of neurobasal media (NBM) was added to the bottom of each well. NCM is conditioned by primary neuronal cells isolated from p0 stage mice. Thus, NCM contains all sorts of molecules, namely a host of neurotrophins including NT-3, NT-4, NGF, and BDNF. On the other hand, NBM is the essential media for neuronal cell culture and contains no cytokines such as neurotrophins. After the WM9 CDK5WT cells were seeded and both the roscovitine and either NCM or NBM were added, the transwell plate was set an in incubator and the assay was allowed to progress for 17 hours. After this time lapse, any cells that were able to migrate across the Matrigel membrane adhered to the bottom of the membrane. This allowed for these invaded cells to be fixed and stained, allowing for clear visualization and quantification of the invaded cells. A representative image of an instance of high

invasion versus an instance of low invasion can be seen in figure 4. After capturing images of the invaded cells, the number of invaded cells was quantified by cell counting with the ImageJ software.

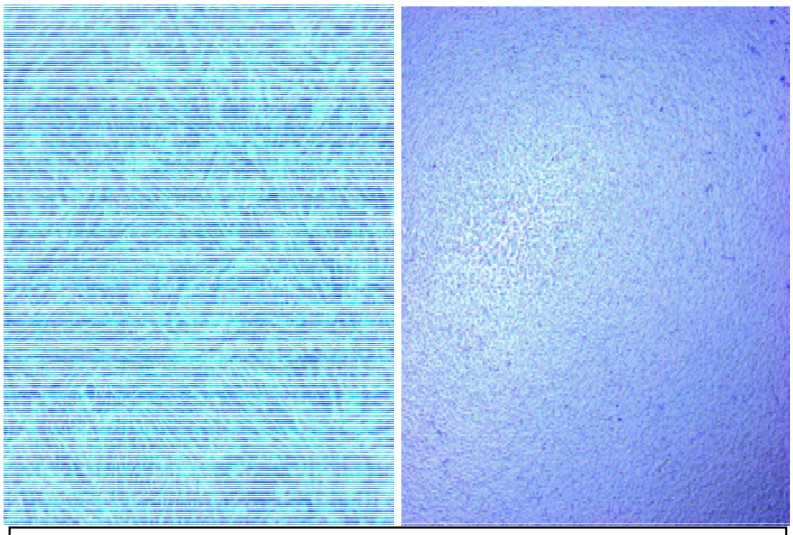

Figure 4 depicts representative images of instances of high invasion (on the left) versus low invasion (on the right) in a Matrigel transwell assay.

Experiment 2

Similar to the first experiment described above, the second experiment in this study utilized a transwell assay mimicking a brain microenvironment in conjunction with a unique method of knocking down CDK5 expression. In order to reduce CDK5 expression, and therefore CDK5 activity, WM9 human metastatic melanoma cells were transduced with the pGIPZ lentiviral vector containing CDK5shRNA for gene knockdown, GFP for transduction tracking, and a puromycin resistance marker to allow for specific selection of successfully transduced cells. In order to accomplish this, plasmids from E.Coli expressing CDK5 were isolated via MaxiPrep. Then 293T packaging cells were plated at $1x10^6$ cells per 10cm plate. After the 293T cells were seeded, a transfection mix including the isolated CDK5 plasmids were added along with shRNA, PEI, and serum free media. This combination allowed for the 293T cells to

produce a viral supernatant containing lentiviral particles with CDK5shRNA, GFP, and puromycin resistance markers. After overlaying the transfection mix on the medium of each plate containing 293T packaging cells for 40 hours, the viral supernatant was collected. 1ml of viral supernatant and 3µL of Polybrene were added with to each well in a six well plate that was seeded with $4x10^5$ WM9 human metastatic melanoma cells. After 40 hours of incubation, the WM9 cells were observed for GFP expression; an indication of successful transduction. Figure 5 shows a representative image of an instance of high lentiviral transduction efficiency.

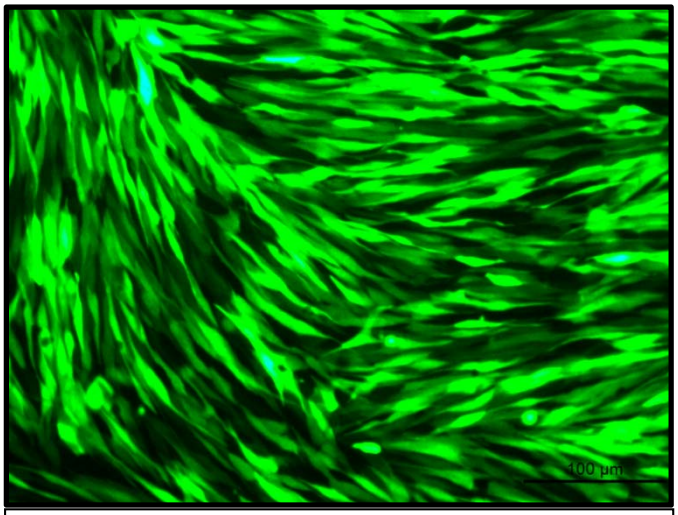

Figure 5 depicts an image of WM9 cells expressing GFP in nearly every cell; a preliminary indication of high efficiency lentiviral transduction.

Finally, cells were selected with puromycin and the successful transduction of CDK5shRNA into the WM9 cells was confirmed via western blotting.

Following lentiviral transduction, WM9 CDK5shRNA cells were utilized in a transwell assay similar to the one described in experiment 1. Figure 6 depicts a schematic illustrating the design of the

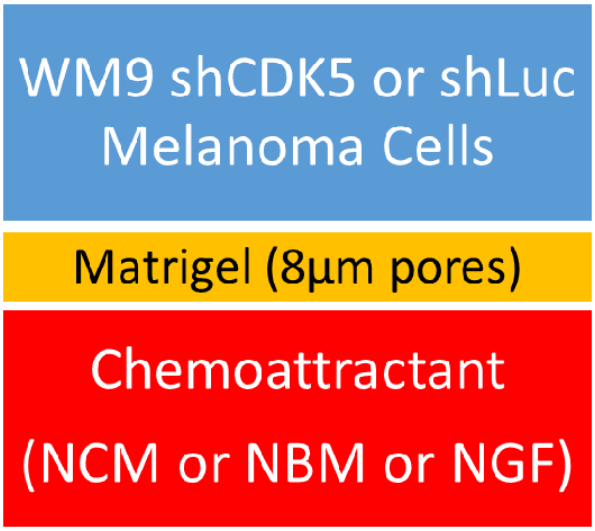

Figure 6 depicts the conditions that were used for the transwell assay in experiment 2. In the top half of each well, WM9 CDK5shRNA or WM9 shLuc (control) cells were seeded. The bottom half of each well contained either NBM, NCM, or NBM + NGF)

transwell assay used in experiment 2. In this transwell assay, similar methods of preparation were used compared to those used in experiment 1. However, the top half of each well was seeded with either WM9 CDK5shRNA or WM9 shLuc cells, which functions as a control vector. In the bottom half of each well, NCM, NBM, or NBM + NGF was added as a chemoattractant. Methods for quantification of invaded cells were analogous to those seen in experiment 1.

Results

Experiment 1

The results from the transwell assay described previously for experiment 1 can be seen in Figure 7.

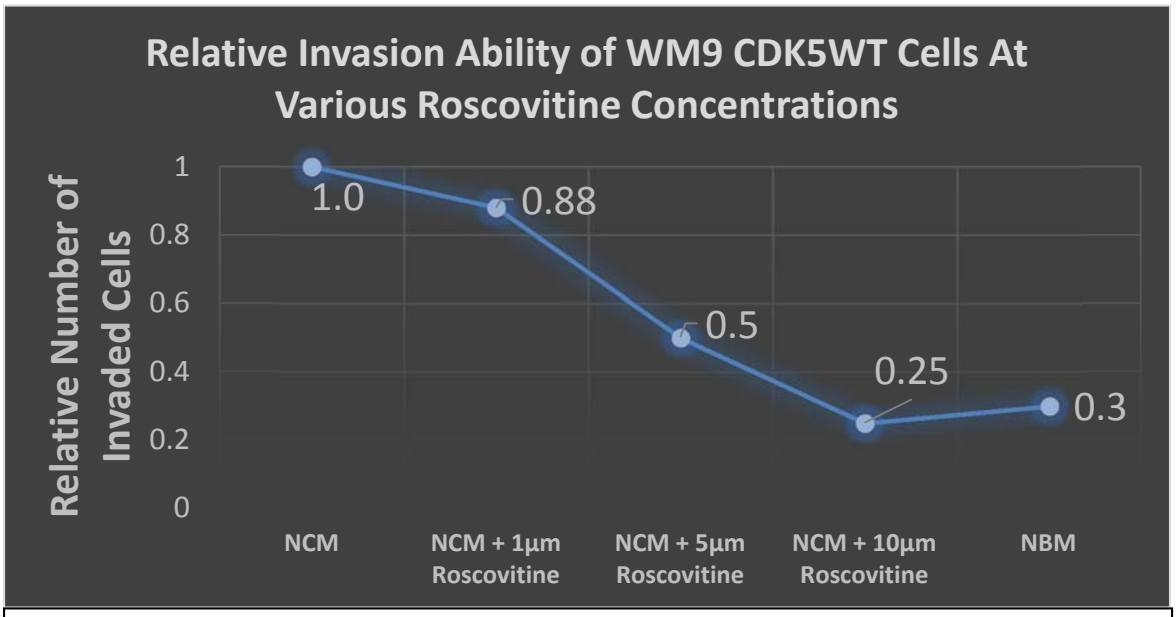

Figure 7 depicts the results from experiment 1.

The vertical axis represents the number of cells that successfully invaded into the brain micro-environment relative to each condition. The highest invasion was seen in the case of NCM as a chemoattractant with no roscovitine added, and thus this condition was set at 1.0. Increasing concentrations of roscovitine resulted in progressively lower levels of invasion. Relative to the no roscovitine condition, treatment with 1µM roscovitine resulted in a 12% decrease in invasion, treatment with 5µM roscovitine resulted in a 50% decrease in invasion, and treatment with 10µM roscovitine resulted in a 75% decrease in invasion. NBM was used as a control and demonstrated 70% lower invasion when compared to NCM with no roscovitine.

Experiment 2

The results from the lentiviral transduction and subsequent transwell assay described in experiment 2 are depicted in Figures 8 and 9, respectively.

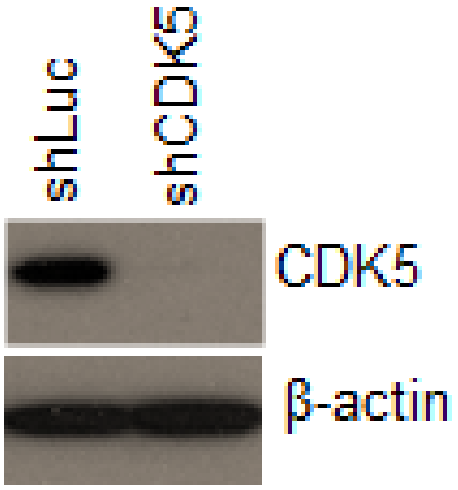

Figure 8 depicts the Western Blotting data following lentirviral transduction of WM9 cells with CDK5shRNA.

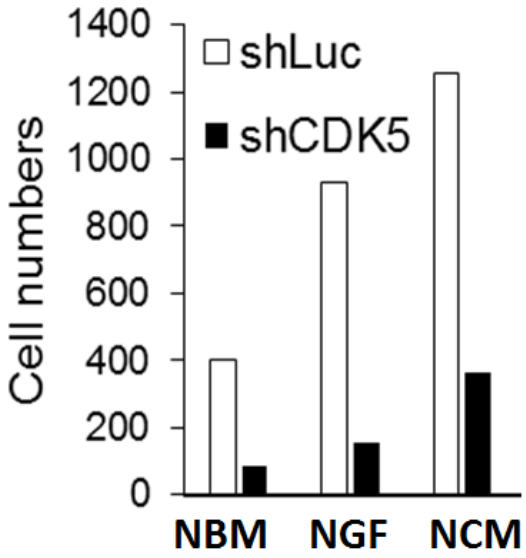

Figure 9 depicts the results from the transwell assay described in experiment 2.

As can be seen in Figure 8, lentiviral transduction of the WM9 cells with CDK5shRNA resulted in a severe decrease in CDK5 expression compared to the control. Thus, the WM9 CDK5shRNA cells demonstrated a significantly reduced invasion ability in the transwell assay regardless of the chemoattractant used, as can be seen in Figure 9. Additionally, the condition in the transwell assay utilizing NCM as a chemoattractant showed the highest invasion ability. Notably, conditions utilizing only NBM as a chemoattractant showed reduced invasion compared to conditions utilizing NBM + NGF as a chemoattractant.

Discussion

Ultimately, the data generated from both experiments 1 and 2 support our central hypotheses that CDK5 is a vital mediator of WM9 metastatic melanoma cell invasion into a brain micro-environment in response to neurotrophin stimulation. In experiment 1, drug induced inhibition of CDK5 showed significant reduction in the ability of WM9 cells to invade a brain micro-environment. Thus, this data provides evidence that CDK5 is a promising therapeutic target for patients with BMM, particularly considering that neurotrophins play a significant role in the event of brain metastasis. It is possible that roscovitine induced inhibition of CDK5 effects cell viability moreso than invasion propensity, however this effect would still be beneficial to patients with BMM if it could be targeted specifically to melanoma cells.

Data generated from experiment 2 provides further evidence that CDK5 is a relevant therapeutic target for patients with BMM. If tumor cells could be specifically transduced with CDK5shRNA, additional avenues for treatment options for BMM may open. On a separate note, the data generated from experiment 2 supports existing research that neurotrophins are responsible for melanoma brain metastasis. This notion is derived from the fact that the chemoattractant of NBM + NGF resulted roughly twice the invasion compared to just NBM as a chemoattractant. Thus, neurotrophins must be at least partially responsible for promoting metastatic melanoma cells to invade a brain microenvironment. Additionally, this data indicates that neurotrophin induced invasion ability is bolstered when multiple types of neurotrophins, such as both NGF and NT-3, stimulate the cell.

Future studies should seek to further elucidate intermediates in the BRAF signaling pathway and their role in brain metastatic melanoma. In this way, additional promising therapeutic targets may be revealed. Furthermore, further studies should utilize a brain metastatic melanoma model in mice so that the relevance of CDK5 in BMM can be explored in vivo, thus taking another step towards bringing new treatment options to the clinic for patients with this dreadful disease.

References

1. Tsao, H., Atkins, M.B. & Sober, A.J. Management of cutaneous melanoma. *N Engl J Med* 351, 998-1012 (2004).
2. Bhatia S, Tykodi SS, Thompson JA. Treatment of metastatic melanoma: an overview. Oncology (Williston Park) 2009;23:488–96
3. Denkins, Y., *et al.* Brain metastases in melanoma: roles of neurotrophins. *Neuro Oncol* 6, 154-165 (2004).
4. Kenchappa RS, Tran N, Rao NG, Smalley KS, Gibney GT, Sondak VK, Forsyth PA. Novel treatments for melanoma brain metastases. Cancer Control. 2013 Oct;20(4):298-306. Review. PubMed PMID: 24077406.
5. McWilliams RR, Rao RD, Buckner JC, et al. Melanoma-induced brain metastases. Expert Rev Anticancer Ther. 2008:8(5):743-755.
6. Yano S, Shinohara H, Herbst RS, et al. Expression of vascular endo-thelial growth factor is necessary but not sufficient for production and growth of brain metastasis. Cancer Res. 2000;60(17):4959-4967.9.
7. Shaffrey ME, Mut M, Asher AL, et al. Brain metastases. Curr Probl Surg. 2004;41(8):665-741.
8. Kienast Y, Von Baumgarten L, Fuhrmann M, et al. Real-time imaging reveals the single steps of brain metastasis formation. Nat Med. 2010;16(1): 116-122.
9. Kang M, Fujimaki T, Yano S, et al. Biology of brain metastases. In: Ali-Osman F, ed. Brain Tumors. Totowa, NJ: Humana Press Inc; 2005:91-106.

10. Agarwala SS, Kirkwood JM, Gore M, et al. Temozolomide for the treatment of brain metastases associated with metastatic melanoma: a phase II study. J Clin Oncol. 2004;22(11):2101-2107.
11. Fidler IJ. The role of the organ microenvironment in brain metastasis. Semin Cancer Biol. 2011;21(2):107-112.
12. Rades D, Panzner A, Dziggel L, et al. Dose-escalation of whole-brain radiotherapy for brain metastasis in patients with a favorable survival prognosis. Cancer. 2012;118(15):3852-3859.
13. Talantov D, et al. (2005) Novel genes associated with malignant melanoma but not benign melanocytic lesions. (Translated from eng) Clin Cancer Res 11(20):7234-7242 (in eng).
14. Abdullah C, Wang X, & Becker D (2011) Expression analysis and molecular targeting of cyclin-dependent kinases in advanced melanoma. (Translated from eng) Cell Cycle 10(6):977-988 (in eng).
15. Parry D, et al. (2010) Dinaciclib (SCH 727965), a novel and potent cyclin-dependent kinase inhibitor. (Translated from eng) Mol Cancer Ther 9(8):2344-2353 (in eng).
16. Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Sciente, Vol.237, pp. 1154-1162.
17. Segal, R.A. (2003). Selectivity in neurotrophin signaling: theme and variations. Annu Rev Neurosi, Vol.26, pp. 299-330.
18. Carlo Pincelli, Katiuscia Dallaglio and Francesca Truzzi (2011). Melanoma and the Nervous System – Novel Pathways Mediated by Neurotrophins and Their

Receptors, Breakthroughs in Melanoma Research, Dr Yohei Tanaka (Ed.), ISBN: 978-953-307-291-3, InTech, DOI: 10.5772/22053.

19. Marchetti, D.; Denkins, Y.; Reiland, J.; Greiter-Wilke, A.; Galjour, J.; Murry, B.; Blust, J. & Roy, M. (2003). Brain-metastatic melanoma: a neurotrophic perspective. Pathol Oncol Res, Vol.9, pp. 147–158
20. Wang, J.J.; Tapinat, A.; Ethell, D.W.; Testa, M.P. & Bredesen, D.E. (2000). Phosphorylation of the common neurotrophin receptor p75 by p38beta2 kinase affects NF-kappaB and AP-1 activities. J Mol Neurosi, Vol.15, pp. 19-29.
21. Gershon, T.R.; Oppenheimer, O.; Chin, S.S. & Gerald, W.L. (2005). Temporally regulated neural crest transcription factors distinguish neuroectodermal tumors of varying malignancy and differentiation. Neoplasia. Vol.7, No.6, pp.575-84.